Amendments to the Drawings:

The attached replacement sheets of drawings include changes to Figs. 1 and 2 and replace the original sheets including Figs. 1 and 2.

In Fig. 1, the identification number "8" at the left side of the sequence listing has been changed to correctly indicate "6" for SEQ ID NO:6.

In Fig. 2, the identification of number "10" at the left side of the sequence listing has been changed to correctly indicate "8" for SEQ ID NO:8.

Attachments following last page of this Amendment:

Replacement Sheets (2 pages)
Annotated Sheet Showing Change(s) (2 pages)

REMARKS

Claims 16, 26-33, 37, 38, and 40-58 are pending in this application. Claims 41-50 are withdrawn pending rejoinder if product claims are allowed. Claims 16, 26-32, 38, and 40 are amended. Figs. 1 and 2 have been amended to indicate correctly the SEQ ID NO each sequence represents. The specification has also been amended to correlate correctly sequences with their SEQ ID NOs.

Claims 16, 26-32, 38, and 40 have been amended to delete reference to SEQ ID NOs: 4, 6, and 8. It is out understanding that claims 32 and 53 are in condition for allowance and allowance is requested.

New Matter

Claims 52 and 54 are rejected under 35 U.S.C. § 112 as presenting new matter. The specification is amended herein to recite "covalent or aggregate conjugates with other chemical moieties, e.g., PEGylation." Such an amendment is proper under 37 C.F.R. § 1.57(f). An analogous example to this situation is provided in MPEP 608.01(p) I .A. Example 2, in which an amendment to the claims was not supported under 37 C.F.R. § 1.57(d) by a foreign patent reference, but the foreign reference did provide support for an amendment to the specification under 37 C.F.R. § 1.57(f), and the amendment to the specification supported the amendment to the claims. In the present case, the PEGylation amendment to claims 52 and 54 are allegedly new matter, *i.e.*, they are not supported by a non-patent publication under 37 C.F.R. § 1.57(d). However, analogously to MPEP 608.01(p) I .A. Example 2, the solution is the above amendment to the specification under 37 C.F.R. § 1.57(f), which incorporates support for the addition of PEGylation to claims 52 and 54. The material being inserted is the material previously incorporated by reference and the amendment contains no new matter. Claims 52 and 54 are supported by the specification as amended.

Furthermore, with respect to the incorporation of support for PEGylation, the specification indicates at page 16 that derivatives include "glycosylation variants, and covalent

or aggregate conjugates with other chemical moieties" and furthermore that "[c]ovalent derivatives can be prepared by . . . standard means." The specification then goes on to cite to three different texts including the Lundblad and Noyes (1988) reference, which provide background techniques for preparing covalent derivatives including PEGylated derivatives. These texts are incorporated by reference in their entirety. PEG is a *chemical moiety* that, through PEGylation, is *covalently* attached to a polypeptide. PEGylation is a very well known technique as evidenced by the fact that PEGylation has been around for at least 30 years, and those of skill in the art are well versed in synthesizing and utilizing PEGylated polypeptides.[1] In this case, the incorporated references are cited with sufficient specificity for covalent derivatives and methods of making covalent derivatives. As those of skill in the art are readily aware that PEGylation is a technique for forming covalent derivatives and PEGylation is taught by the cited references, PEGylation is adequately identified by the present specification and the Lundblad and Noyes (1988) disclosure of PEGylation provides support for the amendment to the specification.

This amendment is believed to satisfy the examiner's concerns and the Applicants request that this rejection be withdrawn.

35 U.S.C. § 112, first paragraph (Enablement)

Claims 16, 26-31, 33, 37, 38, 40, 51-52, and 55-57 are rejected under 35 U.S.C. § 112, first paragraph for allegedly failing to enable claims to an isolated polypeptide comprising at least 17, 20, 25, 30, 35, 50, or 75 amino acids of SEQ ID NO:2, or variants of SEQ ID NO:2. Claim 16 has been amended to recite "[a]n isolated polypeptide comprising a *fragment* of at least 17 contiguous amino acids from SEQ ID NO:2, wherein the *fragment* binds to WSX-1/TCCR." This amendment is believed to satisfy the Examiner's concerns and fully overcome the rejection (independent claim 40 has been similarly amended). Claim 38 has been amended to delete the comprising language and to state that the claimed polypeptide is "at least about 90% identical to SEQ ID NO:2."

[1] *See* Abushowski et al., J. Biological Chem., 282:3582-3586 (1977) (copy enclosed).

The Applicants respectfully point out that the test for enablement is whether experimentation alleged to be necessary is undue, *not whether any experimentation is necessary*. *In re Angstadt*, 537 F.2d 498, 504 (CCPA 1976). When the art typically engages in a type of experimentation, that experimentation is not considered undue. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). In this case, one of skill in the art would merely need to perform a competitive binding assay to see if the selected polypeptide fragments or variants compete with the full length polypeptide of SEQ ID NO:2 in binding to WSX-1/TCCR. Thus, it is easily within the skill of a skilled artisan to generate (*see, e.g.*, p. 31, lines 10-16) and identify (*see, e.g.*, p. 33-36 and 54 (line 12)-55 (line 9)) the claimed polypeptides with the recited functionality without undue experimentation, especially considering the polypeptides are limited to those within SEQ ID NO:2 or the polypeptides are limited to those having 90% identity to SEQ ID NO:2 and given the teaching of the specification regarding detection of WSX-1/TCCR binding. The Applicants respectfully request reconsideration and withdrawal of the enablement rejection in view of the claim amendments.

35 U.S.C. § 112, first paragraph (Written Description)

Claims 16, 26-31, 33, 37, 38, 40, 51-52, and 55-57 are rejected under 35 U.S.C. § 112, first paragraph for allegedly containing subject matter not adequately described in the specification. As discussed above, claims 16, 38, and 40 have been amended to recite polypeptides with specific lengths or percent identities, wherein the polypeptides bind to WSX-1/TCCR.

As established by the Court of Appeals for the Federal Circuit, the standard regarding what is or is not supported by the specification has been clearly articulated as requiring the specification to convey with reasonable clarity to those of skill in the art that, as of the filing data sought, the inventor was in possession of the invention, *i.e.*, whatever is now claimed. *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64 (Fed. Cir. 1991). Further, MPEP § 2163.02 states that "[t]he subject matter of the claim need not be described literally (*i.e.*, using the same terms in

haec verba) in order for the disclosure to satisfy the description requirement." In this case, the Applicants have provided sufficient information.

Specifically, the claims are directed to SEQ ID NO:2 and polypeptides thereof. SEQ ID NO:2 encodes a 242 amino acid polypeptide the complete amino acid sequence of which is listed in the sequence listing as well as being shown in Fig. 1. Several specific sections, i.e., polypeptides, of SEQ ID NO:2 are identified at p. 8 as follows:

> A predicted signal cleavage site may exist between about residues 25-30 of SEQ ID NO: 2; helix A is predicted to run from about residues 33-38 to about residues 54-59 of SEQ ID NO: 2; helix B is predicted to run from about residues 85-90 to about residues 111-116 of SEQ ID NO: 2; helix C is predicted to run from about residues 121-126 to about residues 154-159 of SEQ ID NO: 2; and helix D is predicted to run from about residues 201-206 to about residues 228-233 of SEQ ID NO: 2.

With respect to the disclosed sequences including SEQ ID NO:2, the specification further indicates at p. 10 that "[t]hese amino acid sequences . . . are important in providing sequence information for the cytokine allowing for distinguishing the protein antigen from other proteins and exemplifying numerous variants. Moreover, the peptide sequences allow preparation of peptides to generate antibodies to recognize such segments . . . ." Additionally, a polypeptide is defined at p. 11 to include

> a significant fragment or segment, and encompasses a stretch of amino acid residues of at least about 8 amino acids, generally at least about 12 amino acids, typically at least about 16 amino acids, preferably at least about 20 amino acids, and, in particularly preferred embodiments, at least about 30 or more amino acids, e.g., 35, 40, 45, 50, 60, 75, 100, etc. Such fragments may have ends which begin and/or end at virtually all positions, e.g., beginning at residues 1, 2, 3, etc., and ending at, e.g., 150, 149, 148, etc., in all practical combinations. Particularly interesting peptides have ends corresponding to structural domain boundaries, e.g., helices A, B, C, and/or D.

Further, in addition to other portions of the specification, synthetic methods for producing polypeptides and polypeptide fragments of SEQ ID NO:2 are disclosed at p. 31. Finally,

Example XII. D. shows that IL-27 binds to WSX-1/TCCR. Thus, the polypeptides and polypeptide fragments that bind to WSX-1/TCCR as claimed are well-defined by SEQ ID NO:2. Given the fact that SEQ ID NO:2 is provided, as well as methods for polypeptide synthesis and screening, one of skill in the art would clearly perceive a defined set of peptide fragments.

The Office Action also specifically addresses the 90% identity element of claim 38 and indicates that the claim is allegedly not described because Example 14 of the Guidelines only indicates 95% identity is patentable. Claim 38 has been amended to recite an isolated polypeptide at least about 90% identical to SEQ ID NO:2, wherein the polypeptide binds to WSX-1/TCCR. Support for at least about 90% identity is found at page 13, lines 25-26 of the specification. Example 14 of the Guidelines uses the example of 95%, but there is no limitation in the Guidelines, MPEP, or case law that finds this example to be an absolute limit. When applied to claim 38, the analysis performed in Example 14 of the Guidelines requires a determination whether the single species of SEQ ID NO:2 that binds to WSX-1/TCCR is representative of a genus with 90% structural identity to SEQ ID NO:2 when each polypeptide binds to WSX-1/TCCR. As amended, the claimed polypeptide includes those polypeptides having 90% identity to SEQ ID NO:2 and bind to WSX-1/TCCR, thus the genus is limited.

In summary, the sequence of SEQ ID NO:2 is provided, methods for synthesizing peptides are disclosed and well known in the art, and methods of making and using the claimed polypeptides are also provided, one of skill in the art would consider the subject matter of the claims clearly articulated by the specification. For these reasons, the Applicants respectfully request reconsideration and withdrawal of the written description rejection.

Related Patent

The Applicants call the Examiner's attention to the following related patent: U.S. 7,148,330 (filed November 30, 2001). The patent and file history are available in PAIR and the Examiner is encouraged to review them.

Conclusions

For the reasons set forth above, the Applicants submit that the claims of this application are allowable. Reconsideration and withdrawal of the Examiner's rejections are hereby requested.

In the event that a telephone conversation could expedite the prosecution of this application, the Examiner is requested to call the undersigned at 404-892-5005.

Enclosed herewith is a Request for Continued Examination. The required fees are being charged to Deposit Account 06-1050. Please apply any other charges or credits to deposit account 06-1050.

Respectfully submitted,

Date: October 25, 2007

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Effect of Covalent Attachment of Polyethylene Glycol on Immunogenicity and Circulating Life of Bovine Liver Catalase*

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Methoxypolyethylene glycols of 1900 daltons (PEG-1900) or 5000 daltons (PEG-5000) were covalently attached to bovine liver catalase using 2,4,6-trichloro-s-triazine as the coupling agent. Rabbits were immunized by the intravenous and intramuscular routes with catalase modified by covalent attachment of PEG-1900 to 43% of the amino groups (PEG-1900-catalase). The intravenous antiserum did not yield detectable antibodies against PEG-1900-catalase or native catalase, as determined by Ouchterlony and complement fixation methods, whereas the intramuscular antiserum contained antibodies to both PEG-1900-catalase and catalase. PEG-1900 did not react with either antiserum. Catalase was prepared in which PEG-5000 was attached to 40% of the amino groups (PEG-5000-catalase). This catalase preparation did not react with either antiserum. PEG-1900-catalase retained 93% of its enzymatic activity; PEG-5000-catalase retained 95%. PEG-5000-catalase resisted digestion by trypsin, chymotrypsin, and a protease from *Streptomyces griseus*.

PEG-1900-catalase and PEG-5000-catalase exhibited enhanced circulating lives in the blood of acatalasemic mice during repetitive intravenous injections. No evidence was seen of an immune response to injections of the modified enzymes. Mice injected repetitively with PEG-5000-catalase remained immune competent for unmodified catalase, and no evidence of tissue or organ damage was seen.

We report in a previous paper (1) that covalent attachment of polyethylene glycol to bovine serum albumin renders the protein incapable of eliciting antibody to itself or unmodified albumin.[1] PEG-albumin shows extended circulating life in the blood. In this paper we report the covalent attachment of PEG to bovine liver catalase, and studies on the enzymic properties, immunogenicity, and blood circulating life of the modified enzyme.

EXPERIMENTAL PROCEDURES

Bovine liver catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) was obtained from Sigma Chemical Co. Cyanuric chloride was obtained from Aldrich Chemical Co. and was recrystallized twice from anhydrous benzene immediately before use. Monomethoxypolyethylene glycols of 1900 and 5000 daltons (Carbowax 2000 and 5000) were supplied by Union Carbide. Trypsin, chymotrypsin, and protease (from *Streptomyces griseus*) were obtained from Sigma.

Acatalasemic mice were the kind gift of Dr. Robert N. Feinstein, Argonne National Laboratory. The preparation of 2-*O*-methoxypolyethylene glycol-4,6-dichloro-s-triazine ("activated PEG") has been described (1). Protein was determined by the biuret procedure, and primary amino groups according to Habeeb (2).

Attachment of Activated PEG to Catalase—Reactions were carried out at 4°. To 500 mg of catalase in 50 ml of 0.1 M sodium tetraborate, pH 9.2, was added with constant stirring 4.3 g of activated PEG-1900, an amount 10-fold in excess of available amino groups. The solution was maintained at pH 9.2 by means of a pH-stat (Radiometer, Copenhagen). After 1 h, unattached PEG-1900 was removed by dialysis against 0.01 M phosphate, pH 7.3, using an Amicon ultrafiltration apparatus and the XM-50 membrane. Catalase was reacted in a similar manner with a 10-fold excess of activated PEG-5000. PEG-1900-catalase showed a 43% modification of amino groups by attachment of PEG, with retention of 93% of the original enzymatic activity, while PEG-5000-catalase had a 40% modification of amino groups, and 95% activity.

A series of PEG-1900-catalase preparations was made in which increasing fractions of the amino groups were substituted with PEG-1900. Treatment of catalase with activated PEG-1900 in molar amounts equal to 1, 3, and 7 eq of the available amino groups resulted in products with 13, 19, and 37% of the amino groups substituted by PEG-1900, respectively.

Assay Methods—Catalase and PEG-catalase preparations were assayed according to the method of Beers and Sizer (3) in which the disappearance of peroxide is followed spectrophotometrically at 240 nm. Blood catalase was assayed by the procedure of Feinstein (4). Activity is expressed as perborate units (millimoles of perborate hydrolyzed in 5 min).

Immunological Procedures—Antisera were prepared against catalase and PEG-1900-catalase in New Zealand White, adult female rabbits as described earlier for native and modified bovine serum

studies are reported on PEG-1900-catalase preparations with varying percentages of modification, the percentage of modification is given in parentheses, *e.g.* PEG-1900-catalase (19%).

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‡ Recipient of a predoctoral fellowship from the Charles and Johanna Busch Memorial Fund.

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[1] The abbreviations used are: albumin, bovine serum albumin; PEG-1900, monomethoxypolyethylene glycol of 1900 daltons; PEG-5000, monomethoxypolyethylene glycol of 5000 daltons. PEG-1900-catalase, the catalase preparation with PEG-1900 attached covalently to 43% of its amino groups; PEG-5000-catalase, catalase with PEG-5000 attached to 40% of the available amino groups. When

albumin (1). Complete Freund's adjuvant was used in all intramuscular injections. One week after the completion of the immunization schedule the animals were bled by cardiac puncture and the sera was stored at −20°.

The antisera were tested in gel diffusion slides for *in vitro* precipitating activity. Slides (1.0% agarose in 0.01 M phosphate-buffered saline, pH 7.3, with 1.0% sodium azide) were incubated overnight at room temperature.

Complement fixation (5) was performed using antisera dilutions of 1/100 and 1/200 and antigen concentrations of 100 to 0.39 μg/ml.

In Vivo Circulation Studies – Acatalasemic mice were injected thrice weekly at 2-, 2-, and 3-day intervals in the tail vein with 100 perborate units of catalase, PEG-1900-catalase, or PEG-5000-catalase, for a period of 90 days. The circulating life of injected catalase was measured at 1, 30, 60, and 90 days (immediately following the 1st, 13th, 26th, and 39th injection).

Proteolytic Digestion – Catalase or PEG-5000-catalase, 1.6 mg of either, was digested at room temperature with 5 mg of trypsin, 5 mg of chymotrypsin, or 10 mg of protease in a total volume of 1 ml. Aliquots were taken at various time intervals and assayed spectrophotometrically for activity. The relatively large amounts of enzymes were used because of resistance of PEG-5000-catalase to digestion.

RESULTS

pH Optima of Catalase and PEG-1900-Catalase – The activity of PEG-1900-catalase appears to be quite similar to that of native catalase (Fig. 1). At the extremes of pH (4.5 to 6, 8 to 9.5), PEG-1900-catalase retains somewhat greater activity, which suggests a slight resistance to denaturation. Exposure to pH 12 for 1 min at 20° caused complete inactivation of both catalase and PEG-1900-catalase. At this pH, catalase dissociates into subunits (6).

Effect of Modification of Thermal Stability – The stabilities of catalase and PEG-catalase were tested by two methods. The first method involved holding the enzymes at specific temperatures for 5 min, followed by rapid cooling to room temperature, and assay. The results, shown in Fig. 2, indicate that both catalase and PEG-1900-catalase begin to denature at about 55°. PEG appears to have no effect on stabilization to high temperatures. By contrast, Marshall and Rabinowitz (7) report that covalent attachment of dextran to catalase yields a dextran-catalase conjugate that is more resistant to heat denaturation than native catalase.

In the second method, catalase and PEG-5000-catalase were assayed at various temperatures by the perborate method (Fig. 3). Catalase shows maximum activity at a temperature of about 40°, which is several degrees higher than that shown by PEG-5000-catalase. This suggests that PEG attachment causes some destabilization of the catalase structure. A second point of interest is the increase in activity of both catalase and PEG-5000-catalase as the temperature approaches zero. Such behavior may be characteristic of the perborate assay. Published rate constants for the decomposition of hydrogen peroxide as a function of temperature do not show this change (8).

Proteolytic Digestion of Catalase and PEG-Catalase – Catalase incubated with trypsin showed a rapid decrease in activity, with total loss of activity after 40 min (Fig. 4*A*). This is in agreement with published results (9). PEG-5000-catalase, conversely, lost activity very slowly. After 150 min, 90% of the original activity remained. Chymotrypsin did not inactivate catalase as rapidly as trypsin (Fig. 4*B*). After 60 min, 30% activity was lost. PEG-5000-catalase was virtually unaffected, retaining 98% activity. Catalase lost 90% of its activity upon digestion by *Streptomyces griseus* protease for 60 min (Fig. 4*C*), while PEG-5000-catalase lost 20% of its activity. This protease has a wide specificity (10), which may account for its comparatively greater activity against PEG-5000-catalase than trypsin and chymotrypsin.

Effect on Antibody Production of Covalent Attachment of Polyethylene Glycol to Catalase – Initial experiments were performed using PEG-1900. Catalase was modified to varying degrees (13, 19, 37, and 43%) with PEG-1900. These preparations were used to immunize rabbits by either intravenous or intramuscular injection.

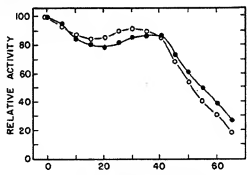


FIG. 3. Activities of catalase and PEG-5000-catalase at various temperatures. Samples were assayed at the various temperatures by the perborate method of Feinstein (4). ●——●, catalase; ○——○, PEG-5000-catalase.

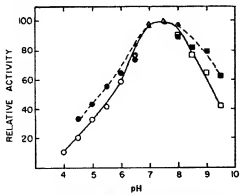


FIG. 1 (*left*). Effect of pH on the activity of catalase and PEG-1900-catalase. Five microliters of a 3 mg/ml solution of either catalase (*open symbols*) or PEG-1900-catalase (*closed symbols*) was diluted in 100 ml of the appropriate buffer and immediately assayed by the spectrophotometric method. ○ or ●, 0.05 M sodium acetate; △ or ▲, 0.05 M sodium phosphate; □ or ■, 0.05 M sodium borate.

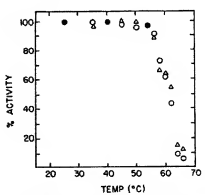


FIG. 2 (*right*). Thermal stability of catalase and PEG-1900-catalase. Samples (1 mg of protein/ml) of catalase or PEG-1900-catalase in 0.01 M phosphate, pH 7.3, were placed in a water bath of specific temperature. After 10 min, the samples were cooled in an ice bath to room temperature and assayed spectrophotometrically. ○, catalase; △, PEG-1900-catalase; ●, values coincide.

By the intravenous route, native catalase elicited a strong immune response (Table I). Anticatalase antiserum reacted with all of the PEG-1900-catalase preparations. Greatest reaction was seen with native catalase and the least with PEG-1900-catalase (43%). Evidently a 43% modification of the amino groups of catalase with PEG-1900 was not sufficient to inhibit the antigen-antibody reaction.

Anti-PEG-1900-catalase (13%) antiserum showed a decrease in antibody production, and the antibody present reacted only with native catalase and 13 and 19% modified PEG-1900-catalase. Antiserum to PEG-1900-catalase (19%) was quite similar to antiserum to PEG-1900-catalase (37%). The reaction with native catalase was weak and one animal from either group did not produce antibody capable of reacting with the modified catalases. There was no detectable reaction between antiserum to PEG-1900-catalase (43%) and any of the antigens.

These results indicate that attachment of PEG-1900 to 43% of the amino groups yielded an adduct that did not elicit antibody production by the intravenous route of administration. When immunization was carried out by the intramuscular route, however, all of the PEG-1900-catalase preparations elicited an immune response. This may be due to denaturation of the enzyme during the homogenization of antigen with adjuvant, and exposure of antigenic determinants, or simply due to the use of adjuvant, which enhances the response.

All of the intramuscularly derived antisera reacted with PEG-1900-catalase (43%). Intramuscularly derived antisera were tested against PEG-5000-catalase. As shown in Fig. 5, PEG-5000-catalase did not react with either anticatalase antiserum or anti-PEG-1900-catalase (43%) antiserum.

Enzyme Replacement Therapy in Acatalasemic Mice—Acatalasemic mice were injected thrice weekly for a period of 90 days. At various time intervals, the blood profile of injected enzyme was determined.

Initial enzyme replacement therapy was performed using PEG-1900-catalase. Blood catalase levels following the first injection are shown in Fig. 6*A*. Catalase decreased to endogenous levels (2 to 3 units/ml) within 12 h. This removal could not be due to the immune response, which requires about 1 week in the mouse. The enzyme may have been taken up by the liver, as has been found in replacement therapy for certain of the lysosomal storage diseases (11). PEG-1900-catalase, conversely, remained active in the blood for 48 h. On Day 30, following the 13th injection, catalase was removed within 2 h, probably due to immune clearance, as antibodies to catalase were present in the serum. In contrast, PEG-1900-catalase showed no indication of immune clearance and circulated as in the first injection (Fig. 6*B*). The circulating life of PEG-1900-catalase after 60 and 90 days of injections is shown in Fig. 6, *C* and *D*. Again, extended blood life is seen, with no evidence of immune clearance. Animals injected with native catalase showed such severe immunological reactions after a few injections that injections were terminated after 60 days.

Replacement therapy was also performed with PEG-5000-catalase. The blood picture after the first injection (Fig. 7*A*) was quite similar to that obtained in the first study. Catalase again was removed rapidly; PEG-5000-catalase remained active in the blood for more than 50 h. It appears that catalase modified with PEG-5000 has a slightly longer blood life than catalase modified with PEG-1900. Injections of PEG-5000-catalase were continued as described for PEG-1900-catalase. On Day 30 (Fig. 7*B*), catalatic activity again disappeared rapidly, while PEG-5000-catalase-injected mice retained blood catalase activity after 52 h.

Injections over periods of 60 days (Fig. 7*C*) and 90 days (Fig. 7*D*) failed to produce substantial changes in the circulating life of PEG-5000-catalase. No antibodies to catalase or PEG-5000-catalase were detectable in serum samples of mice injected with PEG-5000-catalase after 30, 60, and 90 days of injection.

After Day 90, PEG-5000-catalase-injected animals were tested for immune competence against catalase using the Jerne technique (12). Spleen cells sensitized to catalase were detected, indicating that PEG-5000-catalase did not produce immunosuppressive effects.

Pathology—Three mice were submitted for necropsy following 90 days of injection with PEG-5000-catalase. Gross pathology revealed no visible lesions. Histopathological examination of 25 tissues (hematoxylin and eosin stains) showed that all tissues were within normal limits.

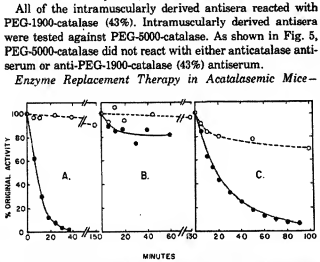


FIG. 4. Proteolytic digestion of catalase (●) and PEG-5000-catalase (○). To 1.6 mg protein in 1 ml of 0.05 M phosphate, pH 7.0, was added either 5 mg of trypsin (*A*), 5 mg of chymotrypsin (*B*), or 10 mg of *Streptomyces griseus* protease (*C*). Aliquots were removed at intervals and assayed spectrophotometrically.

TABLE 1

Immunodiffusion studies using catalase, and catalase modified to varying degrees with PEG-1900, against rabbit antisera to each antigen

Antisera were prepared in rabbit pairs by the intravenous injection of catalase, or catalase to which increasing amounts of PEG-1900 were attached. Center wells contained 10 μl of antiserum and peripheral wells contained 10 μl of a 1 mg/ml of solution of catalase or modified catalase in 0.01 M phosphate-buffered saline, pH 7.3. Results are expressed as estimated relative amount of precipitation for each antiserum-antigen pair. Tests were carried out in quadruplicate. +++ indicates greatest precipitation; – indicates no observable precipitation.

Antiserum to	Antigen				
	Catalase	PEG-catalase (13%)	PEG-catalase (19%)	PEG-catalase (37%)	PEG-catalase (43%)
Catalase	+++/+++	++/++	++/++	+/++[a]	+/+
PEG-catalase (13%)	++/++	++/++	++/+	–/–	–/–
PEG-catalase (19%)	++/+	++/–	++/–	++/–	–/–
PEG-catalase (37%)	+/+	–/++	–/++	–/++	–/++[a]
PEG-catalase (43%)	–/–	–/–	–/–	–/–	–/–[a]

[a] Results checked by complement fixation.

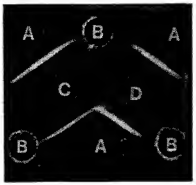
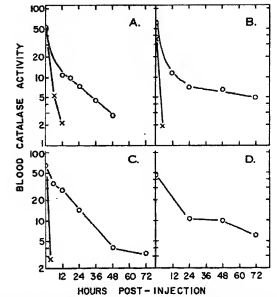


FIG. 5 (*left*). Immunodiffusion plate showing the reaction of catalase (*A*) and PEG-5000-catalase (*B*) against intramuscularly derived anticatalase antiserum (*C*) and intramuscularly derived anti-PEG-1900-catalase antiserum (*D*). Enzyme concentrations were 3 mg of protein/ml in 0.01 M phosphate, pH 7.3. Samples, 20 μl, were added to wells 4 mm in diameter and 6 mm apart.

FIG. 6 (*right*). Blood circulating life of catalase (×) and PEG-1900-catalase (○) in acatalasemic mice. On Day 1 (*A*), 10 animals in each group were injected with 100 perborate units of enzyme by the tail vein; 15 min later, 0.15 ml of blood was taken from the eye orbit from a set of two animals from each group and assayed according to Feinstein (4). Different sets of two animals were used for the 6-, 12-, 24-, and 36-h bleedings. The initial set of two animals was again bled at 48 h. The animals were then placed on a regimen of thrice-weekly injections at 2-, 2-, and 3-day intervals of 100 perborate units of enzyme. The assay was repeated after the 13th injection (Day 30; *B*), the 26th injection (Day 60; *C*) and the 39th injection (Day 90; *D*).



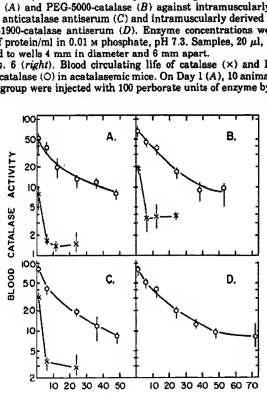


FIG. 7. Blood circulating life of catalase (×) and PEG-5000-catalase (○). Twenty animals were used in each group; individual points represent the average of four animals. Otherwise, conditions are similar to those given in Fig. 6. *Vertical bars* show the range of values; *circles* give the mean.

DISCUSSION

A major objective of the work reported in this and the previous paper (1) has been to develop procedures for reducing or eliminating the immunogenicity of proteins and, in the case of enzymes, also to retain reasonable activity. Good immunogens typically have a rigid, complex surface structure to which antibodies can be made. We rationalized that the covalent attachment of a linear, flexible, uncharged hydrophilic polymer to available but nonessential groups on an enzyme might provide a shell around the enzyme that covers antigenic determinants and, by presenting a flexible, unbranched, hydrophilic surface for inspection by the immune processes, prevent recognition of the interior enzyme as a foreign substance against which an immune response would be provoked. At the same time, the shell would be permeable to the smaller substrates so that enzymatic activity could continue.

PEG was selected for covalent attachment because of its nonimmunogenicity and compatibility with blood (13, 14) and because it best fits the criteria we selected. Dextran, another promising polymer, was rejected because of its known immunogenicity in humans (15, 16). The monomethoxypolyethylene glycols offer the additional advantage of having a single terminal hydroxyl group for activation or modification for coupling purposes.

Catalase has a molecular weight of 242,000 and contains 108 lysine residues (17). The attachment of PEG-1900 to 43% of the free amino groups yields an adduct of about 335,000 daltons. Attachment of PEG-5000 to 40% of catalase amino groups approximately doubles the size of the adduct. These are minimum values, as other groups on the enzyme also may have reacted with activated PEG during the coupling process. The slight decrease in enzymatic activity exhibited by the modified catalases indicates that small molecules have little difficulty penetrating the PEG layer that presumably surrounds the enzyme.

The ease with which experimental animals tolerate repetitive injections of PEG-catalase over extended periods suggests a future for PEG-enzymes in enzyme therapy. Preliminary work in our laboratory has shown that several other enzymes can be modified by PEG attachment without excessive loss of activity and, in the case of one enzyme, uricase, which is being

tested by repetitive injections as described in this paper, we also see extended blood circulating life and absence of apparent immunological effects.

If, by PEG attachment, a substantial percentage of enzymes can be rendered apparently nonimmunogenic and capable of extended circulating life while retaining activity, the way seems opened for large scale expansion of enzyme therapy. For example, enzymes from diverse and inexpensive sources may be used. The investigator, free of concern for adverse immunological effects, designs experiments related to the particular metabolic aspects of the clinical problem with which he or she is dealing. Long term enzyme therapy would seem routine. For alteration of blood metabolites, PEG-enzymes might be injected directly into the blood stream; for storage diseases, incorporation of PEG-enzymes into liposomes (17, 18) or erythrocytes (19, 20) for eventual uptake into lysosomes would seem feasible. PEG-enzymes may prove to be resistant to degradation by lysosomal proteases, as PEG-catalase is to trypsin, chymotrypsin, and *S. griseus* protease.

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